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C-Phycocyanin — the Main Photoreceptor in the Light Dependent Germination Process of *Anabaena* Akinetes

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Abstract. Action spectra obtained for the light dependent germination process of *Anabaena* akinetes, as determined according to different photon flux rate response relationships (24 wavelengths between 400 nm and 750 nm) uniformly demonstrate a maximum between 620 nm and 630 nm. Thus the most active spectral range for the germination process coincides with the maximum of the light absorption by C-phycocyanin. Since germination is only slightly impaired when electron transport is blocked by DCMU, the formation of photosynthetic products as prerequiste of germination is rather unlikely. The participation of phycocyanin in cyclic photophosphorylation and in other photochemical processes is discussed with respect on possible participation in stimulating. There are indications of different flux rate and wavelength dependencies of germination and growth, respectively.

Key words: Cyanobacteria (Blue-Green Algae) – Akinetes – Germination – Action spectrum – Photoreceptor – Phycocyanin.

According to our present knowledge the akinetes of hormogonial Cyanobacteria (= Cyanophyceae) represent resting cells which are formed only under certain conditions (Wolk, 1973) and which are more resistant to various extreme conditions in the environment than the cells of the filaments from which they originate (see Fogg et al., 1973; Nichols and Carr, 1978).

Akinetes are altered in size (Fjerdingstad, 1969), DNA- (Ueda and Sawada, 1972), pigment- (Fay, 1969a), water- (Braune and Sanke, 1979) and fatty acid content (Yamamoto, 1972), the wall condition (Miller and Lang, 1968; Dunn and Wolk, 1970), metabolism (Fay, 1969b) and the accumulated metabolic products (see Carr and Whitton, 1973).

The mechanisms leading to an activation of physiological processes during germination and outgrowth are practically unknown. Although it has long been recognized that apart from nutrient supply (Glade, 1914) the presence of light is an essential precondition for germination (Harder, 1917), attempts to explain the cause of this light dependence have remained restricted. From the observation that light must act for a long time during the germination process Harder first suggested the role of photosynthesis. This concept was supported by the fact that the action of light on akinetes of *Nostoc punctiforme* could be replaced by some organic compounds, especially by saccharose. However, the akinetes germinated also in an atmosphere free of CO_2 and under such low light intensities that photosynthetic products could not be expected. Therefore, although the author preferred the "hypothesis of photosynthesis" as an interpretation of the germination process he discussed a "matter transforming action of light": „Man kann annehmen, daß bei Vorhandensein der übrigen Keimungsbedingungen im Innern der Zelle ein Stoffumsatz stattfindet, der nichts mit der Assimilation der Kohlensäure zu tun hat, aber nur in Gegenwart des Lichtes möglich ist". Kaushik and Kumar (1970) concluded from their experimental results with colour foils "that spore germination does not require photosynthetic light". Reddy et al. (1975) reported a red/far red photoreversal for germination similar to phytochrome action in comparable activating processes in mosses, ferns, and higher plants. On the other hand, the germination process was said to be blocked by the photosynthesis inhibitor DCMU, again an argument for the role of photosynthesis (Yamamoto, 1976).

These conflicting views and the attraction of investigating such morphogenetic phenomena at the

Abbreviations. DCMU = 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PPO = 2,5-diphenyloxazole; POPOP = 1,4-bis(2,5-phenyloxazolo)-benzene; dpm = disintegrations per min; $N\lambda$ = photon flux rate (nmol · cm^{-2} s^{-1}) at the wavelength λ (nm); "irradiance" = fluence rate (W · m^{-2}); PC = C-phycocyanin; Chl = chlorophyll a.

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lowest level of evolution made it worthwhile to analyse the role of light in the germination process of Cyanobacterial akinetes in greater detail.

By establishing action spectra, we first intended to obtain more exact information about the spectral ranges active in germination and thereby also about potential photoreceptors. Additionally, it was attempted to assess experimentally the role of photosynthesis during germination by direct measurements of ^{14}C-incorporation during this process.

Material and Methods

Akinetes of the filamentous Cyanobacterium *Anabaena variabilis* (Kützing) strain JS-07 isolated from river water were investigated. On agar medium (nutrient medium No. 6, Gromov, 1965; 1,5% agar) and in continuous light (8 $W \cdot m^{-2}$, white fluorescent, 22 $\pm$ 2° C) the organism converts more than 80% of its cells into akinetes within 2 weeks.

In the experiments we used akinetes from cultures 12 weeks old which are previously stored for 8 weeks in dim light (1 $W \cdot m^{-2}$). Akinetes were purified by filtration and fractionated centrifugation of the raw material suspended in sterile nutrient solution, and adjusted to about 10^6 cells per ml. Aliquots of this suspension were spread on a thin agar layer, enclosed in damp chambers (on microscope slides) and irradiated on these agar layers. By this means both outgrowth and growth were ensured for a few days. Contamination with heterotrophic bacteria was neglectible. The minimal size of the experimental object (average cell dimensions 17 $\times$ 8 µm) allowed us to irradiate large quantities of cells simultaneously using a microscope. The light source, collector lens, diaphragm and condenser were of such dimensions that all points in the object plane were irradiated at the same intensity. 12 V/100 W Halogen lamps (VEB Narva) were used as a light source. For selecting spectral ranges (between 400 nm and 750 nm; some wavelengths are specified in Fig. 3) we used interference filters type IF and SIF (VEB Carl Zeiss Jena) with half-band width between 5 and 10 nm and 30–45% peak transmittances. Interference filters were checked for the absence of second order transmission using the Carl Zeiss spectrophotometer "Specord".

Irradiance was measured with a vacuum thermopile Vth-20-W (ZOS der AdW der DDR, Abt. Strahlungsempfänger) in connection with the DC Millipicometer MV 40 (VEB Präzitronik, Dresden) in W $\cdot m^{-2}$. The radiometer has a relative sensitivity of 6,4 V/W and was calibrated to a standard value by the Amt für Standardisierung und Meßwesen der DDR, Berlin. It has a flat spectral response over the wavelength range used. The irradiance in the microscope beam was measured on the level of the objects. The area of the radiometer window was smaller than the area irradiated on the microscope slide.

Monochromatic irradiation was adjusted by neutral filters NG (VEB Jeanaer Glaswerke Schott & Gen.) in connection with the aperture diaphragm to constant values of the photon flux rates ($N\lambda$) of 0.5, 1 and 2 nmol $\cdot cm^{-2} s^{-1}$, uniformly used in the experiments. For 1 nmol $\cdot cm^{-2} s^{-1}$ the incident energy fluence attained between 3 $W \cdot m^{-2}$ (when λ = 404 nm) and 1,6 $W \cdot m^{-2}$ (when λ = 750 nm). Additionally, heat absorbing filters with nearly homogenous transmission in the selected spectral range (type C 9971) were used. Therefore, the temperature at cell level was not essentially higher than that of the irradiation laboratory (25 $\pm$ 1° C).

Monochromatic irradiation (48–72 h, continuously) was started after a dark period of 24 h. During exposure, the number of germinated akinetes and the length of the hormogonia were monitored photographically in 5–7 analyses used the portable microphotographic equipment mf-matic BA 2 (VEB Carl Zeiss Jena). These photographs were analysed later with the microfilm documentation equipment DOCUMATOR DL 2 together with the electromechanical particle counter LEUCONOR 2 (VEB Rathenower Optische Werke). The relative length of filaments were expressed in growth percentage terms compared with the mean value of the akinetes; the value of 10 in this sense corresponds to about 25 µm. It was therefore possible to determine simultaneously the reaction of the object in 10 different sets of irradiation apparatus while excluding all further light during the period of irradiation. At the same time this procedure enabled subjective errors to be reduced to a minimum. Each single evaluation comprised 200–300 akinetes and was repeated 4 times in parallel experiments.

^{14}C-incorporation rates were determined on 500 µl aliquots of akinete suspension (about 10^6 cells per ml) on membrane filters (Synpor 3, Chemapol Prag; mean pore size 1.5 µm) floating on 15 ml of standard nutrient solution in gas-tight glass vessels. Into the nutrient solution was injected 150 µl $NaH^{14}CO_3$-solution (5 µCi; specific activity 0.1 mCi $mmol^{-1}$, Isocommerz Berlin) at various times during pre-irradiation (i.e. at different phases of germination) at 700 lx, with mixed ("white") light at 22 $\pm$ 2° C. The process was stopped after 4 h exactly, the filters maintained for 10 min above fuming HCl and after drying and resolution the radioactivity was determined (Liquid Scintillation Counter LKB Wallac 81,000; scintillant: 3 l toluene, 2 l methylcellusolv, 20 g PPO, 2 g POPOP, 400 g naphthalene). After correcting for dark fixation and control, the activity is expressed as dpm. Information about DCMU concentrations represent final concentrations in the incubation medium.

Results

After 30–70 h of irradiation 90–96% of the cells have been germinated. The bursting of the akinete wall is a well defined criterion for precisely determining the event of germination. After storage in continuous darkness germination was not observed and, up till now, no replacement for light has been found. Concequently the akinetes of *Anabaena variabilis* obligatorily require light for germination.

Response to Varied Light Intensities

Figure 1 shows the percentage of germinated akinetes after 48 h and 72 h respectively in mixed ("white") light as a function of increasing fluence rates. The optimum range lies between 5 and 20 $W \cdot m^{-2}$. Above 25 $W \cdot m^{-2}$ the outgrowth is increasingly inhibited. The lower limit of stimulation is very low: even at intensities of 0,1–0.3 $W \cdot m^{-2}$ some of the akinetes are able to germinate within 2 days, i.e. at energies which are insufficient for filament growth (growth in 250 h: at 0.3 $W \cdot m^{-2}$ 40 µm, at 16 $W \cdot m^{-2}$ 520 µm).

Germination as a Function of Wavelength

The spectral dependence of the germination process in 24 selected wavelength ranges between 404 nm and 750 nm (cf. Fig. 3) and with 3 different photon flux

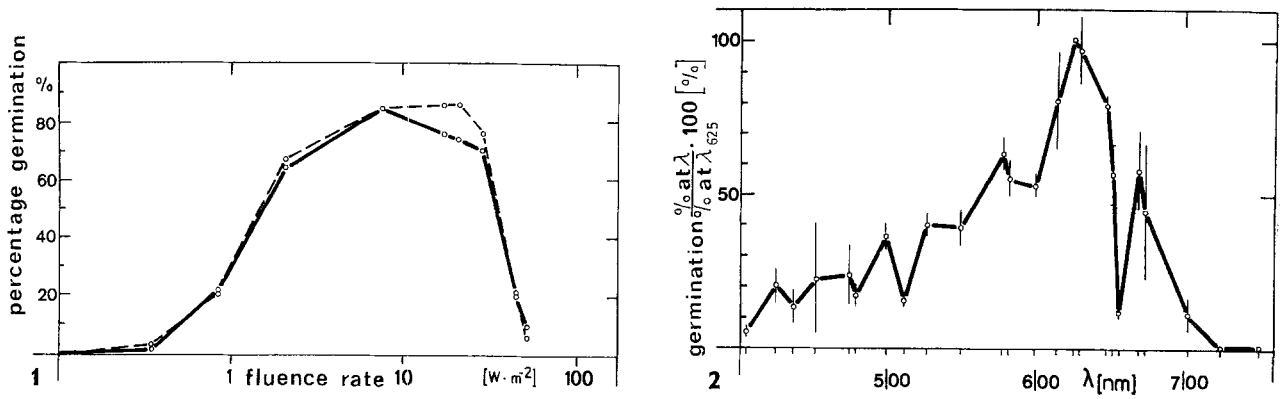

Fig. 1. Intensity dependence of the germination of akinetes (%) in mixed ("white") light 48 h (——) and 72 h (– – –), respectively, after beginning of irradiation (26 ± 4° C)

Fig. 2. Efficiency of different wavelengths (λ_{nm}) for the germination of *Anabaena* akinetes (as percentage relative to the percentage of germination at $\lambda_{625\,nm}$; error bars: one standard error). Uniform flux rate: 1 nmol · cm^{-2} s^{-1}

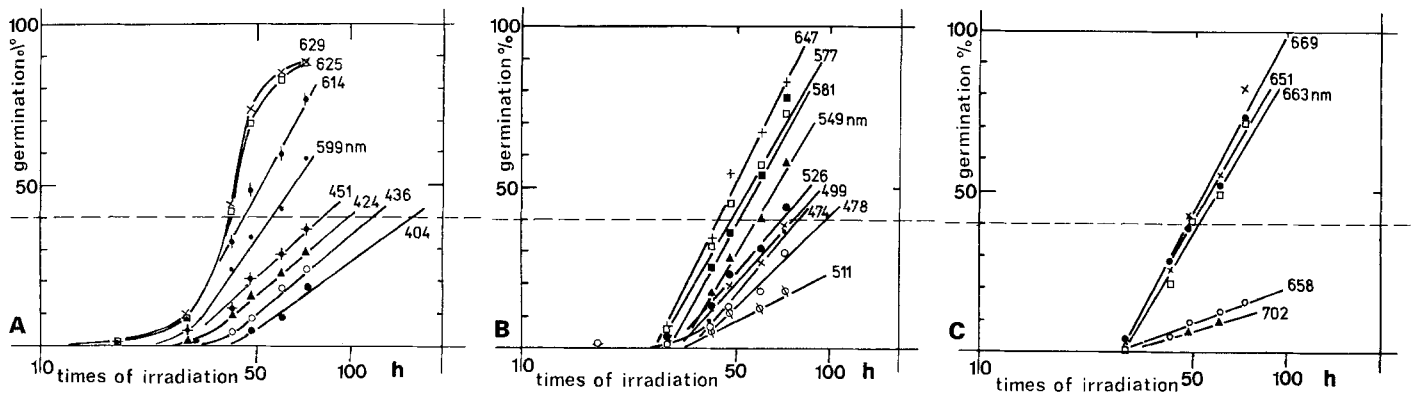

Fig. 3A—C. Germinative efficiency of irradiation with different wavelengths (nm) using varied irradiation times (h) and a constant photon flux rate of 1 nmol · cm^{-2} s^{-1} (dose response curves)

rates in each case (0.5, 1, 2 nmol · cm^{-2} s^{-1}) was determined. Fig. 2 shows the result in the case of 1 nmol · cm^{-2} s^{-1}; the proportion of germinated akinetes at each wavelength being expressed as a percentage of the value at λ 625 nm (each circle represents the mean value of germination at 4–6 points in time between 22 and 66 h after beginning of irradiation). The curves obtained independently are basically very similar: all show a distinct major peak at 620–630 nm and a minor peak at 670 nm. It is difficult to interpret whether a true stimulation also takes place at 577 nm because, although the curves tended to decline towards the shorter wavelength, the decline itself was very irregular.

If the germination response at varied spectral ranges is plotted against duration of irradiation at a constant photon flux rate, "dose" response curves (cf. Hartmann and Cohnen Unser, 1972; Shropshire, 1972) are obtained which can be used in case of a sufficient linearity between 40 h and 70 h of irradiation (Fig. 3A–C in the case of 1 nmol · cm^{-2} s^{-1}) for construction of an action spectrum. For this purpose we selected 40% germination as a standard level of photoresponse. The light dose (D_{40}) required to induce this effect was plotted as a relative quantum efficiency D_{40} at 625 nm/D_{40} at λ in % against wavelength (Fig. 4). The action spectrum obtained in this way demonstrates (like the wavelength dependence of germination in Fig. 2) the characteristicts of absorption spectra of living akinetes (cf. Fig. 7): the major peak at 625 nm coincides with the absorption maximum of PC, the peak at 670 nm corresponds with the red absorption region of Chl. The shoulder at 580 nm obviously belongs to the absorption spectrum of PC; light absorbed in this region is also active in initiating germination. On the other hand, the quanta absorbed by Chl and carotinoids in the blue range are scarcely effective.

To analyse reciprocity relationships, those periods of irradiation (t) which give the same percentage of germination (mean values at all λ selected) are com-

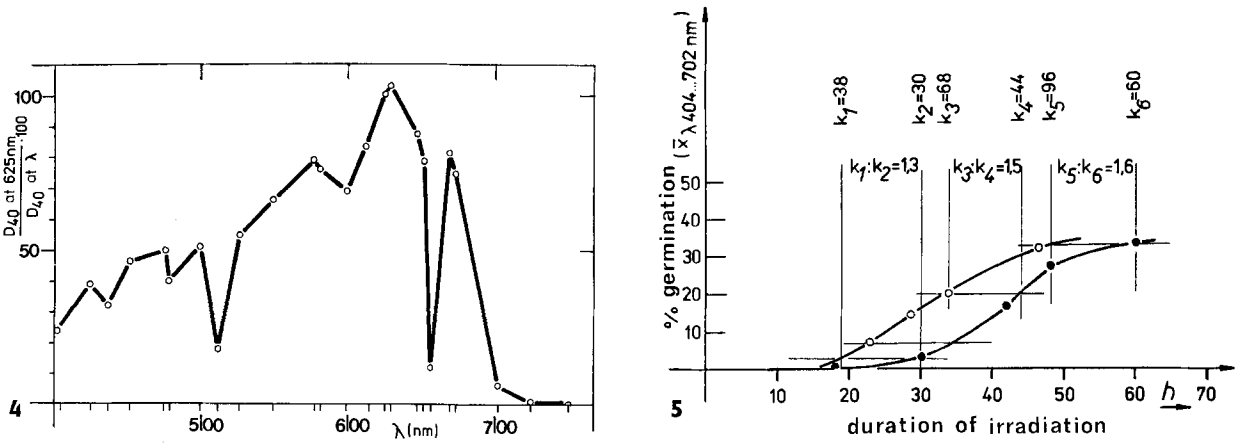

Fig. 4. Action spectrum for germination of akinetes, calculated from data of Fig. 3A–C (see text for details)

Fig. 5. Percentage germination (mean values of all λ examined) as a function of duration of irradiation (h) at photon flux rates of 1 (●——●) and 2 nmol · cm^{-2} s^{-1} (○——○). $I \times t = k$

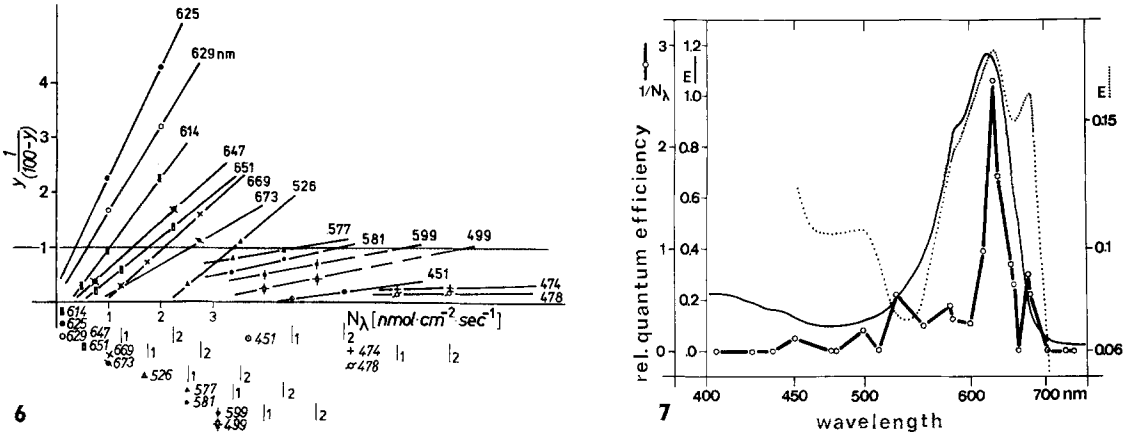

Fig. 6. Photon flux response curves for germination of *Anabaena* akinetes (linear approximations for some representative wavelengths, λ_{nm}). The efficiency $Y \cdot \frac{1}{(100-Y)} = 1$ equals 50% germination, Y = percentage germination

Fig. 7. Action spectrum for germination of akinetes (○——○) calculated from data of Fig. 6 determined for relative quantum efficiency of 1. N_λ equals the photon flux rate (nmol · cm^{-2} s^{-1}) required to induce this relative efficiency. For comparison, the spectrum of C-phycocyanin (phosphate buffer, pH 7.0) (———) and the in vivo spectrum of a single akinete (·····) are plotted against the same wavelength scale

pared with one another at photon flux rates of 1 and 2 nmol · cm^{-2} s^{-1} (I). Quotients from the products $I \times t$ were not 1 but in fact relatively constant at 1.3–1.6 over a large period of irradiation (Fig. 5). The predominant role of PC in the absorption of light quanta evoking germination can be demonstrated more impressively with an action spectrum obtained by plotting the relative quantum efficiency $1/N_\lambda$ (calculated from photon flux rate response curves, shown for some representative wavelengths in Fig. 6) against wavelengtht (Fig. 7). N_λ represents the photon flux rate required to produce a relative effectiveness of 1 (corresponding to 50% germination) following the relationship $Y \cdot \frac{1}{100-Y}$ (for satisfactory transformation into straight lines), Y = percentage germination.

Growth of Hormogonia as a Function of Wavelength

Growth processes following the outgrowth of the cells from the akinete envelope are also wavelength dependent. The increase in relative length of hormogonia for all investigated wavelengths between 400 nm and 750 nm (photon flux rate 1 nmol · cm^{-2} s^{-1}) shows differences compared with germination (Fig. 8): the major peak of effectiveness of monochromatic irra-

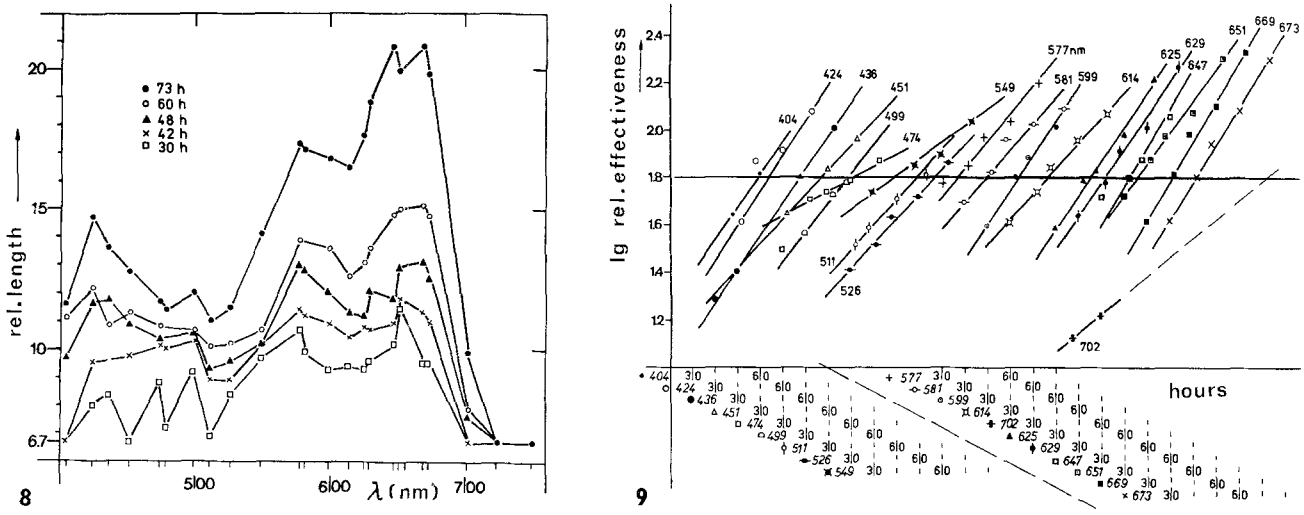

Fig. 8. Elongation of hormogonia as a function of irradiation with different wavelengths at a photon flux rate of 1 nmol · cm^{-2} s^{-1} (the relative length of 10 units corresponds to about 25 µm). All akinetes which are germinated at the moments indicated (hours after the beginning of irradiation) were measured

Fig. 9. Relative elongation (lg percentage of growth) of hormogonia at irradiation (1 nmol · cm^{-2} s^{-1}) with different wavelengths (nm) as a function of duration of irradiation (h)

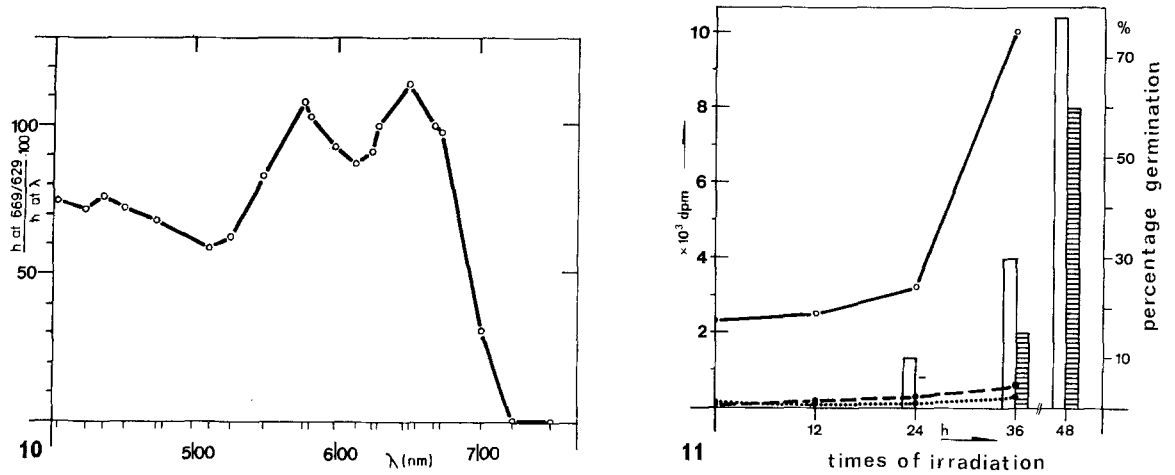

Fig. 10. Action spectrum of hormogonia elongation determined for a relative growth of 1.8 (see text for details) calculated with data from Fig. 9

Fig. 11. Net-incorporation of ^{14}C during germination of akinetes (h after beginning of irradiation) influenced by 10^{-4} M DCMU. DCMU present throughout the total duration of the experiment (·····) and only during the 4 h period of exposure to $NaH^{14}CO_3$ (– – – –) respectively; control without DCMU (———). Columns: Percentage of germinated akinetes (shaded: influenced by 10^{-4} M DCMU)

diation is clearly shifted to the long-wave region and blue light also promoted the elongation of filaments.

Different growth rates for all wavelenghts obtained by varied duration of irradiation with constant photon flux rate (1 nmol · cm^{-2} s^{-1}) are plotted for all wavelenghts in Fig. 9 as "dose"-response curves. They are used to establish the action spectrum (Fig. 10): relative quantum efficiency is expressed as the duration of irradiation (h) at 1 nmol · cm^{-2} s^{-1} necessary for a relative growth of 1.8 (about 63% elongation; as a percentage of 629 nm and 669 nm, respectively).

Also in this case, the activity of light absorbed by PC is clearly diminished as compared with that of Chl. The shoulder at about 580 nm, already observed in connection with germination, is noteworthy. Observations are not available for other photon flux rates and the results therefore cannot be generalized.

Effect of Inhibited ^{14}C-Incorporation on Germination

The role of photosynthesis in the germination process was examined by inhibiting carbon incorporation dur-

ing the pregerminative phase. As is demonstrated by Fig. 11, this is easily achieved using 10^{-4} M DCMU. While the rate of carbon incorporation was reduced to about 5% 36 h after the beginning of irradiation, the number of germinated akinetes decreased to about 50% only. After 48 h the reduction was merely 20%. The relative insensitivity of germination of *Anabaena variabilis* akinetes to DCMU was corroborated by 5 independent series of experiments. The germinated cells either died immediately or after no more than 1–2 cell divisions.

Discussion

Action spectra for the light dependent germination process of Cyanobacteria has not hitherto been demonstrated. The results of our experiments with *Anabaena* akinetes show unmistakable maximum effectivity for quanta absorbed by PC (λ_{max} at 620–630 nm). This might support interpretations by Harder (1917) and Yamamoto (1976) which supposed photosynthetic products as a cause of germination: the action spectra are similar to those of photosynthesis in Cyanobacteria which "invariably show that light absorbed by chlorophyll a is virtually ineffective" (Stanier and Cohen-Bazire, 1977). However, germination of akinetes in our experiments with DCMU-inhibited photosystem II activity indicate that this is rather unlikely, confirming results which demonstrated that increased dry matter content of germinating akinetes is not a necessary precondition for outgrowth (Braune and Sanke, 1979). Therefore other possibilities of light action in connection with PC as photoreceptor for the germination process are to be taken into account: (1) the role of PC in the energy yielding process of photosystem I (cyclic photophosphorylation) and (2) an eventual regulative function of the pigment comparable with the phytochrome action.

ATP synthesis in Cyanobacteria seems indeed to be linked primarily with the cyclic electron transport (Duane et al., 1965; Bottomley and Stewart, 1976). Also Bornefeld and Simonis (1974) and Bornefeld (1976) came to the same conclusion from the observation that the ATP-pool in *Anacystis nidulans* was only slightly impaired by DCMU in the presence of light. However, because a direct participation of PC in photosynthetic electron transport has been repeatedly excluded (Duane et al., 1965; Arnon et al., 1974) only transfer of the absorbed light energy to photosystem I would be its function in this process (see Stanier, 1977). On the other hand the significance of such an energy supply for the germination process of akinetes has not been demonstrated till now. Results of Kaushik and Kumar (1970) and Reddy et al. (1975) implicate the possibility of a non-photosynthetic light action. There is as yet no evidence in Cyanobacteria for the occurence of phytochrome, the photomorphogenetic pigment responsible for similar photomorphoses in eukaryotic plants. The closed chemical relationship of the chromophores of both pigment systems and the detection of photochromic phycobiliproteins (Scheibe, 1972; Kendrick and Spruit, 1977; Björn, 1978) which can be responsible for photoconvertible photomorphoses in Cyanobacteria (Bogorad, 1975), however, permits us to suppose that such a regulative function might also be possible. Particularly after Björn and Björn (1976) succeeded in isolating 3 photochromic phycobiliproteins ("phycochromes"), one of them obviously identical with the f-type and s-type chromophore of PC, such a speculation might be of considerable relevance to the problem under discussion: it points the way to a comparison with the phytochrome action at this low level of evolution where phycobiliproteins, besides their known function in energy supply systems, might in addition fulfil such signal function. The hitherto submitted observations on such a reversible steering process (Reddy et al., 1975) are, however, insufficient to assume such a mechanism for light dependent germination of Cyanobacterial akinetes, but call for further investigation.

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